

# Characterization and pharmacological properties of in vitro propagated clones of *Echinacea tennesseensis* (Beadle) Small

Rita M. Moraes · Hemant Lata · Joko Sumyanto ·  
Ana M. S. Pereira · Bianca W. Bertoni · Vaishali C. Joshi ·  
Nirmal D. Pugh · Ikhlas A. Khan · David S. Pasco

Received: 6 October 2010 / Accepted: 17 January 2011 / Published online: 1 February 2011  
© Springer Science+Business Media B.V. 2011

**Abstract** Tissue culture techniques have been used to establish and maintain a repository of medicinal *Echinacea*. In vitro clones obtained from hypocotyls of germinated seeds, varied macroscopically, microscopically and exhibited variation in immune enhancing activity. Two in vitro produced clones of *Echinacea tennesseensis* (Beadle) Small (ETN 03 and ETN 11) were identified as high and low activity based on the activation of human monocytes. Phenotypic analyses of ETN 03 and ETN 11 clones were done using AFLP (Amplified Fragment Length Polymorphism) assay. Results of the AFLP assay revealed that no mutation has occurred during in vitro multiplication, storage, and acclimatization into soil. Plants of ETN 03, ETN 11 clones were cultivated for two growing seasons. Extracts of their dry leaves and roots exhibited immune enhancing activity; however, the variation in activity

noticed between clones during micropropagation diminished and was no longer statistically relevant.

**Keywords** *Echinacea tennesseensis* · Germplasm · AFLP · Microscopy · HPLC · Endophytes

## Introduction

*Echinacea* is a well accepted herbal supplement in the United States with annual sales in 2006 of \$129 million (Tilburg et al. 2008). The public acceptance of *Echinacea* as herbal supplement is due to its immune-stimulant and anti-inflammatory properties. Several clinical studies on the therapeutic use of *Echinacea* have demonstrated effects on recovery or prevention of infections (Giles et al. 2000; Lindenmuth and Lindenmuth 2000), while other trials have failed to show efficacy (Mark et al. 2001; Melchart and Linde 1999) such as the work reported by Turner et al. (2005), which concluded that the use of root extracts of *Echinacea*, either alone or in combination, showed no clinical effect on the treatment of rhinovirus infection.

*Echinacea* products are sold in a variety of formulations containing different plant parts of three *Echinacea* species (*E. angustifolia* DC, *E. pallida* (Nutt) Nutt. and *E. purpurea* (L) Moench), which contribute to great variability among products. In addition, there is no cultivation and processing methods established to assure the quality of *Echinacea* products for consumers. These factors highlight the importance of characterizing *Echinacea* active constituents and the need to establish production methods. Senchina et al. (2006) studied ethanol tinctures of 18 months old, dried *Echinacea* roots and their capability to retain cytokine modulating properties during storage. The study demonstrated that different *Echinacea* species

R. M. Moraes (✉) · H. Lata · J. Sumyanto ·  
V. C. Joshi · N. D. Pugh · I. A. Khan · D. S. Pasco  
National Center for Natural Products Research,  
School of Pharmacy, The University of Mississippi,  
University, Oxford, MS 38677, USA  
e-mail: rmoraes@olemiss.edu

R. M. Moraes · J. Sumyanto · I. A. Khan  
Center of Water and Wetland Resources, The University  
of Mississippi Field Station, 15 Road 2078, Abbeville,  
MS 38601, USA

A. M. S. Pereira · B. W. Bertoni  
Plant Biotechnology Center, The University of Ribeirao Preto,  
Av. Costabile Romano 2201, Ribeirao Preto, SP 14096-380,  
Brazil

I. A. Khan · D. S. Pasco  
Department of Pharmacognosy, School of Pharmacy,  
The University of Mississippi, University, Oxford,  
MS 38677, USA

have different patterns of immune modulation and efficacy in treating viral infections. Further examining ethanol tinctures of *E. tennesseensis* on proliferation of blood mononuclear cells, Senchina et al. (2009) concluded that activity vary by plant organ with roots having the strongest activities.

Using a different extraction procedure, Pugh et al. (2008) reported that the majority of in vitro monocyte/macrophage activation exhibited by *Echinacea* plant material was due to bacterial lipoproteins and lipopolysaccharides (LPS) present in the extract. According to Tamta et al. (2008) variations in the amount of these bacterial products were responsible for the 200-fold difference in the activity of commercially diverse bulk *Echinacea* material. The study also suggested that bacterial components present in *Echinacea* extracts were not acquired during postharvest manipulation.

According to Lata et al. (2006) endophytic bacteria were isolated from in vitro repository of *Echinacea* sp and these in vitro clones exhibited differences in immune enhancing activity; EC<sub>50</sub> = 1.6 to >1,000 µg/ml (Pugh et al. 2005). Anticipating that natural diversity within plants is partially responsible for the types of endophytic bacteria, this study investigates host diversity role on *Echinacea* bacterial products and immune enhancing activity. In vitro *E. tennesseensis* was the selected species for the study because the species showed great differences in activity between in vitro clones with less differentiation between groups.

## Materials and methods

### *Echinacea* in vitro repository

The North Central Regional Plant Introduction Station (NCRPIS), in Ames, Iowa provided seeds of *E. tennesseensis* accession PI 631250. All experiments were conducted with shoot cultures obtained from hypocotyl explants. Each germinated seed represented one clone. Seeds were surface disinfected as follows: 1% NaOCl (20% v/v bleach) and 0.1% Tween 20 for 10 min followed by washing three times in sterile distilled water prior inoculation on the germination media. Aseptic seedlings were initiated on half strength MS medium (Murashige and Skoog 1962).

After 10 days, 0.5–1.0 cm long hypocotyls were taken as explants for the initiation of shoot cultures. According to Sauve et al. 2004, *E. tennesseensis* can effectively be regenerated from hypocotyls, cotyledons, flower stalks and leaf explants. In this study, shoots grew on half strength MS salt medium containing 3% (w/v) sucrose, 0.8% (w/v) agar, and supplemented with 2.2 µM of benzyladenine (BA) per liter. The medium was adjusted to pH 5.7. Shoot

cultures have been maintained for 24 months with transference each 30 days. All cultures were incubated at 25 ± 2°C, 16 h photoperiod under fluorescent light with a photon flux of 52 µmol m<sup>-2</sup> s<sup>-1</sup> following the protocol described by Lata et al. (2003, 2004). Each seedling was considered a different clone and eight clones coded as ETN 01, 02, 03, 04, 06, 09, 11 and 15 were produced.

### In vitro storage of shoot cultures

The effects of low temperature storage on the viability of *Echinacea* in vitro shoot cultures were investigated using MS medium supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar incubated at 5°C and 10°C with 16 h photoperiod under fluorescent light with a photon flux of approximately 52 µmol s<sup>-1</sup> m<sup>-2</sup>. The control treatment was incubation at 25 ± 2°C. Growth was maintained for 8 months without subcultures. The effectiveness of low temperature storage on germplasm conservation was done using two *E. tennesseensis* clones (ETN 03 and ETN 11).

### Plantlet acclimatization

Rooted plantlets of different clones of *E. tennesseensis* were transferred to a soil substrate composed of a mixture (1:2 v/v) potting soil (Potting Mix Miracle Gro 0.14 0.14 0.14) and sand (Garden Basic Play Sand, Sims Bark Co, Tuscumbia, AL). Potted plantlets were maintained under mist-irrigation watering cycle for 1 min every hour during 6 h period similar to the procedure reported for *E. pallida* and *E. angustifolia* (Lata et al. 2003, 2004). Later two different clones designated as ETN 03 and 11 were planted in pots and compared with plants of *E. tennesseensis* produced from seeds purchased from Johnny's Selected Seeds, Winslow, Maine. Seeds were sowed in plastic trays on March 3, 2006, after 2 months seedlings were planted in pots. Plants obtained from seeds were denominated as ETN seedl (Table 4).

### Morphological characterization

Clones of *E. tennesseensis* were observed at different developmental stages. Macro and microscopic examinations were made for all the clones. Morphological features such as shoot length, leaf shape, size and trichome type of clones ETN 03 and ETN 11 are displayed in Table 2. Microscopic examinations were made using Nikon SMZ-U stereo scope, as well as a Nikon Eclise E-600 fluorescence microscope equipped with Kodak DC 2990 Zoom Digital camera. Image processing was done using Adobe photo shop.

**Table 1** Selected primers and level of polymorphism among 13 samples of in vitro produced clones of *E. tennesseensis* for the AFLP analysis

Primer combinations	No of amplified bands	No of polymorphic bands	Ratio of polymorphic bands (%)
E-ATG + M-AGT	43	7	16.27
E-AGT + M-ATT	57	7	12.28
E-ATC + M-AGT	54	3	5.55
E-AGT + M-AT	79	1	1.26
E-AG + M-AT	65	1	1.53
E-AG + M-ATA	85	1	1.17
Total	383	20	5.22
Average	63.8	3.3	5.17

*E* indicates *Eco*RI adapter sequences, *M* indicates *MSe*I adapter sequences

## Genotypic characterization

### DNA extraction

In vitro produced plants derived from seedlings of *E. tennesseensis* PI 631250 accession were propagated using the procedure by Lata et al. [12, 13]. Total DNA was extracted from actively growing shoot cultures of the following clones ETN 02, 09, 11 and 15 at in vitro multiplication phase and from cultures of ETN 11 maintained under low temperature storage. DNA was also extracted from eight *E. tennesseensis* clones (ETN 01, 02, 03, 04, 06, 09, 11 and 15) after acclimatization in the greenhouse.

### AFLP assay

The CTAB (cetyltrimethylammonium bromide) method of Doyle and Doyle (1987) was used to extract total genomic DNA shoots of the samples above described. Leaf samples were freeze-dried and the material was ground into fine powder. Amplified Fragment Length Polymorphism (AFLP) analyses were performed as described by Vos et al. (1995). Briefly, the restriction of genomic DNA (200 ng) was done with two enzymes (*Eco*RI/*MSe*I) at 37°C for 3 h using a thermocycler model PTC™-100 (MJ Research Inc.). The digested fragments were subsequently ligated with *Eco*RI and *MSe*I adapter sequences in a 10.0 µl reaction volume at 23°C, for 3 h. Fragments were pre-amplified by PCR cycles using *Eco*RI and *MSe*I primers with one selective nucleotide (*Eco*RI-A and *MSe*I-A, respectively) using the following cycle profile: (1) 94°C for 2 min; (2) 94°C for 1 min; (3) 56°C for 1 min; (4) 72°C for 1 min; (5) 72°C for 5 min; steps 2–4 were repeated 26 times. The PCR products were diluted four times and stored at –20°C. The six primer combinations used were:

*Eco*-ATG/*MSe*I-AGT; *Eco*-AGT/*MSe*I-ATT; *Eco*-ATC/*MSe*I-AGT; *Eco*-AGT/*MSe*I-AT and *Eco*-AG/*MSe*I-ATA.

The pre-amplified reaction contained 1 µl *Eco*RI + (1 oligo) (25 ng µl<sup>−1</sup>), 1 µl *MSe*I + (1 oligo) (25 ng µl<sup>−1</sup>), 0.8 µl dNTPs 2.5 mM, 2 µl 10× Buffer B (Promega), 1.2 µl MgCl<sub>2</sub> (25 mM), 0.6 µl Taq DNA polymerase (5 ng µl<sup>−1</sup>) (Promega) and 2 µl of bound DNA. The selective amplification was performed using following cycle profile: (1) 94°C for 2 min; (2) 94°C for 1 min; (3) 56°C for 1 min; (4) 72°C for 1 min; (5) 72°C for 5 min; steps 2 and 4 were repeated for 26 times. The products of amplification were diluted 4 times and stored at –20°C. The selective amplification reaction AFLP-PCR contained 1 µl *Eco*RI + (2 or 3 oligo) (25 ng µl<sup>−1</sup>), 1.2 µl *MSe*I + (2 or 3 oligo) (25 ng µl<sup>−1</sup>), 0.4 µl dNTPs 2.5 mM, 2 µl 10× Buffer B (Promega), 1.2 µl MgCl<sub>2</sub>, (25 mM), 0.2 µl Taq DNA polymerase (5 ng µl<sup>−1</sup>) (Promega) and 1.5 µl DNA pre-amplified. After the selective amplification, 8 µl of loading buffer was added to each reaction. The AFLP selective PCR were denatured at 95°C for 5 min and immediately transferred to ice. The AFLP fragments were separated and visualized using 6% polyacryl-amide gel on a sequencing cell, Sequi-Gen GT (Bio-Rad). The gel was stained with a silver nitrate Kit of Promega (Madison, WI, EUA) according to Creste et al. (2001) procedure.

### Data analysis

For this numerical analysis, a matrix with the presence or absence of the bands was built and analyzed using the NTSYS software program (version 2.1; Exeter Software). Jaccard coefficient was used to construct the similarity matrix (Jaccard 1908) transformed into dendrogram by the UPGMA (unweighted pair-group method with arithmetic averages) algorithm. The confidence of the groups was tested applying a bootstrap analysis with 1,000 replicates using FreeTree software (version 0.9.1.50). The average and standard deviation of the similarities observed among individuals was used to calculate genetic diversity with this accession.

## Pharmacological characterization of *Echinacea* clones

### Assay for monocyte activation

Finely ground *E. tennesseensis* plant material (50 mg) was extracted with 0.5 ml of water containing 4% sodium dodecyl sulfate (SDS) at 98°C for 1 h. SDS was removed using SDS-out reagent (Pierce, Rockford, IL) following by addition of 5 volumes of 95% ethanol the extract precipitated, and was then washed with 95% ethanol, air dried at 55–60°C. The precipitate was dissolved in 2%

octylglucoside for analysis. The human monocyte cell line THP-1 (American Type Culture Collection, Manassas, VA) was transfected with a luciferase reporter gene containing two copies of NF-kappa B motif form HIV/IgK, which was previously described by Pugh et al. (2001). Activity of samples is reported as percent of maximal activation achieved with LPS 10 µg/ml (*E. coli*, serotype 026:B6, Sigma). During the extraction procedure the sample is washed with ethanol to eliminate plant components that inhibit monocyte activation (anti-inflammatory), while hot water with detergent (SDS) will favor extraction of bacterial lipoproteins and LPS (Pugh et al. 2008).

#### Clones and seedlings plants grown in pots for immune enhancing activities

Seedlings and clonal propagated plants were planted in pots (10 cm × 10 cm × 34 cm). Pots were filled with same substrate, a mixture of sand and Pro-Mix BRK with mycorrhiza soil (Hummert International Co. 4500 Earth City, MO). After planting, the soil in the pots was covered with wood mulch (Delta Select, Louisiana Soil Product of Ruston LLC, LA). The plants were placed into a complete randomized design and maintained under full sun at the University of Mississippi Biology Field Station. Water was supplied by a dripping system and the applied volume is 200 ml on a daily basis per pot. The experiment was harvested during the first growing season.

To evaluate the effects of *E. tenneensis* genetic diversity on immune enhancing activity, samples were harvested after 24 months of cultivation. After harvest, plants were separated into roots and leaf biomass, washed extensively and freeze-dried. Plant material was ground into a fine powder using a coffee grinder. Data were analyzed using the Procedure of SAS (SAS Institute Inc.; Cary, NC, USA) and means separation was by LSD at  $P \leq 0.05$ . Regression analysis was used to evaluate and compare linear trend over time for immune activity and biomass accumulation.

#### Results and discussion

In vitro produced clones of *E. tenneensis* show different morphological and chemical characteristics

AFLP analysis was performed on 13 individuals representing eight in vitro clones. The analysis revealed a total of 383 amplified scored DNA bands with an average of 63.8 bands per primer combination (Table 1) with a total 6-primer combination. The dendrogram analysis was based on the Jaccard similarity index to separate genotypes and the unweighted pair-group average dendrogram revealed

that ETN 03 and ETN 11 were distant phenotypes within the accession (Fig. 1). These two clones (ETN 03 and ETN 11) were previously selected based on their immune modulator activities. In addition, the AFLP analysis confirmed that no mutation has occurred during the process of in vitro propagation and storage and acclimatization. There was 100% similarity for clones growing under different conditions, a result confirmed by 98 and 99% bootstrap (Fig. 1). Using six primers, AFLP results have shown 5.17% polymorphism, a rate considered low for open-pollinated species, however *E. tenneensis* has a restricted habitat and, to a certain extent, the population has been under genetic erosion imposed over the years. Mechanda et al. (2004) reported the level of similarity in *E. tenneensis* as 94.84% but still reflects that ETN 03 and ETN 11 are distinct clones.

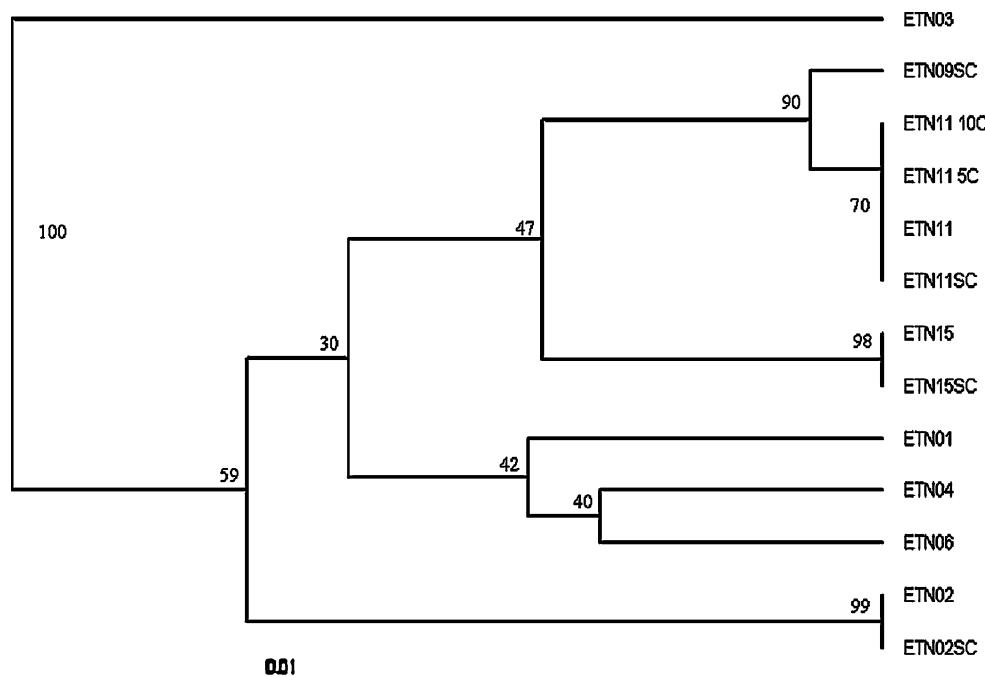
In vitro produced clones were characterized by morphological, chemical and biological analyses during shoot multiplication. Characterization was carried out during acclimatization to the soil to demonstrate phenotypic differences between ETN 03 and ETN 11. Morphological data presented in Table 2 show that the leaf veins, leaf margin and hair density were different characters between ETN 03 and ETN 11 clones through out the growth cycle. During the in vitro stage ETN 11 produced larger leaves with shorter hair length and more number of stomata than ETN 03, the content of chicoric acid in ETN 03 (0.88/100 µg of dry weight) was more than leaves of ETN 11 with 0.25/100 µg of dry weight and ETN 03 immune enhancing activation was over 100-fold greater than ETN 11.

The clones ETN 03 and ETN 11 were produced via organogenesis and later stored in chambers to low positive temperature (5 and 10°C) to evaluate their viability in storage. ETN 03 and ETN 11 resisted well the low temperatures and survived well the 8 month storage period, while cultures at 25°C without subculture have consumed the media showing signs of starvation such as yellowing of the leaves (Table 3). Both clones (ETN 03 and ETN 11) had excellent recovery with 100% survival. Recovery from low temperature storage (5 and 10°C) induced 3 to 7-fold increase on shoot proliferation, while at 25°C only 70% were able to grow (Table 3).

Distinct phenotypes grown under same growth conditions resulted in similar activities in outside pot plantings

The distinct features of ETN 03 and ETN 11 clones did not translate into different immune modulator activities after two growing seasons. Data in Table 4 suggest that diversity within *E. tenneensis* showed no effect on monocyte activation and both plant parts (roots and leaves) have similar pharmacological activities. Contrasting results were

**Fig. 1** UPGMA dendrogram based on the Jaccard similarity index calculated from AFLP analysis of in vitro propagated clones of *E. tennesseensis*. Clones ETN 01, 02, 03, 04, 06, 11 and 15 were planted at greenhouse. Codes ETN 02 SC, ETN 09 SC, ETN 11 SC and ETN 15 SC were in vitro shoot cultures. ETN 11 5C and ETN 11 10C are cultures in storage under 5 and 10°C



**Table 2** Morphological characteristics of *E. tennesseensis* clones (ETN 3 and ETN 11) in the in vitro cultures and after acclimatization in the soil

Characters	In vitro shoot cultures		Acclimatized plants	
	ETN 03 <sup>a</sup>	ETN 11	ETN 03	ETN 11
Shoot length	4.5 cm	5.4 cm		
Leaf shape	Linear-lanceolate	Ovate-lanceolate	Linear-lanceolate	Linear-lanceolate
Leaf length	2.0 cm	2.5 cm	6.0 cm	6.0 cm
Leaf width	0.3 cm	0.6 cm	1.5 cm	1.4 cm
Leaf apex	Acute	Acute	Acute-rounded	Acute
Leaf base	Cuneat	Cuneat	Cuneat	Cuneat
Leaf margins	Undulate	Entire	Undulate	Entire
Leaf veins	Three prominent	Mid-vein prominent	Three prominent	Mid-vein prominent
Hair density	Sparse	Dense	Sparse	Dense
Hair length	900 µm	600 µm	700 µm	500 µm
Hair joints	Slightly swollen	Swollen	Swollen	Swollen
Apex	Acute	Acute	Acute	Acute
Stomata	Cruciferous	Cruciferous	Cruciferous	Cruciferous
GC length <sup>a</sup>	20 µm	30 µm	50 µm	40 µm
No of stomata @ 20×	15	17	5	5
No of sub cells	35	36	16	27
Stomata index	1,542.8	1,747.2	531	518.5

<sup>a</sup> GC = guard cells

described by Senchina et al. (2009); roots have the strongest modulator activity if compared to other plant parts. It is known that different extraction procedures influence the concentration and type of compounds present in the fraction to be tested, thus affecting the medicinal properties. The extracts tested in this study contained bacterial lipopolysaccharides and lipoproteins as previously reported by

Pugh et al. (2008) and Tamta et al. (2008). Furthermore, these studies demonstrated that these bacterial products derived from endophytes.

Even though, the rhizospheres are hot areas of colonization, endophytic bacteria can colonize stems and leaves (Hardoim et al. 2008). The research knowledge on the role of endophytes colonizing flowers, fruits and seeds is limited,

**Table 3** Effects of low temperature incubation on shoot growth of *E. tennesseensis* and culture re-growth after 8 months germplasm storage

Temperature	ETN 03 <sup>a</sup>			ETN 11		
	No of Shoots <sup>b</sup>		Survival (%)	No of Shoots		Survival (%)
	Storage	Re-growth		Storage	Re-growth	
5°C	2.8 ± 0.8	9.6 ± 0.9	100	3.2 ± 0.8	11.0 ± 2.4	100
10°C	3.4 ± 0.5	6.2 ± 0.5	80	5.6 ± 1.5	6.4 ± 1.5	90
25°C	6.0 ± 0.7	4.8 ± 0.8	65	6.4 ± 1.3	5.8 ± 1.1	70

<sup>a</sup> ETN 03 and ETN 11 are code to designate two clones of *E. tennesseensis*

<sup>b</sup> No of shoots is the average of shoots ± standard deviations

**Table 4** Variation in monocyte stimulatory activity exhibited by the extracts of different *E. tennesseensis* phenotypes harvested after two growing seasons

Genotype	Extract of dry plant part (6 µg)		Extract of dry plant part (120 µg)	
	Leaves	Roots	Leaves	Roots
ETN 03 <sup>a</sup>	29.0 <sup>b</sup>	35.0	72.4	65.5
ETN 11	39.3	33.8	76.3	77.5
ETN Seedl.	25.0	32.0	71.0	83.8
LSD 0.05	11.6		17.2	

<sup>a</sup> ETN 03 and ETN 11 are codes to designate two clones of *E. tennesseensis* and ETN Seedl. refers to plant material propagated by seeds

<sup>b</sup> Each value represents the average activity of extracts from four individual plants, with each extract tested in duplicate. Control value for untreated cells was 1.3 ± 0.19

Mastretta et al. (2009) and van Oevelen et al. (2003) demonstrated that the endophytic bacteria were present in seeds of *Nicotina tabacum* and *Phycothria* sp. Seedlings were the source of explants of our in vitro shoot cultures *E. tennesseensis*, and Lata et al. (2006) isolated endophytic bacteria from the cultures after 8 months of cultivation period. These shoot cultures have also shown difference in immune enhancing activity which we have attributed to the presence and latency of endophytic bacteria.

After the plants were cultivated for two growing seasons on similar growing conditions, the extracts show no significant difference in activation. According to Schweitzer et al. (2006), plant diversity is a major factor promoting or limiting bacterial growth and consequently host/bacterial products. However, the high degree of similarity within *E. tennesseensis* allowed association of similar endophytic taxa after the plants were cultivated under similar conditions (Table 4). Thus, it is no surprise that closely related individuals growing under similar conditions would contain similar microflora, and therefore exhibit the same levels of monocyte activation.

In conclusion, this study can be summarized as follows: (1) An in vitro repository of *E. tennesseensis* was established to evaluate immune enhancing activity of *E. tennesseensis*/bacterial products. (2) This protocol is a useful tool for the production of clones with distinct morphological and chemical characteristics; (3) *E. tennesseensis*

diversity was not a major factor influencing in vitro macrophage activation.

**Acknowledgments** This research was partially funded by grants from the National Institutes of Health RO1 AT002360 (NCAAM) to DSP and the USDA, Agricultural Research Service Specific Cooperative Agreement No. 58-6408-7-012.

## References

- Creste S, Tulmann Neto A, Figueira A (2001) Detection of single sequence repeat polymorphism in denaturing polyacrylamide sequencing gels by silver staining. Plant Molec Biol Rep 19:299–306
- Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull 19:11–15
- Giles JR, Palat CT, Chien SH, Chang ZG, Kennedy DT (2000) Evaluation of *Echinacea* for treatment of common colds. Pharmacotherapy 20:690–697
- Hardoim PR, van Overbeek LS, van Elsas JD (2008) Properties of bacterial endophytes and their proposed role in plant growth. Trends Microb 16:463–471
- Jaccard P (1908) Nouvelles recherches sur la distribution florale. Bulletin de la Société vaudoise de Sciences naturelles 44: 223–270
- Lata H, De Andrade Z, Schaneberg B, Bedir E, Khan I, Moraes RM (2003) Arbuscular mycorrhizal inoculation enhances survival rates and growth of micropropagated plantlets of *Echinacea pallida*. Plant Med 69:673–676
- Lata H, Bedir E, Moraes RM, Andrade Z (2004) Mass propagation of *Echinacea angustifolia*: a protocol refinement using shoot

- encapsulation and temporary immersion liquid system. *Acta Hort (ISHS)* 629:409–414
- Lata H, Li XC, Silva B, Moraes RM, Halda-Alijia L (2006) Identification of IAA-producing endophytic bacteria from micropropagated *Echinacea* plants using 16S rRNA sequencing. *Plant Cell Tiss Org Cult* 85:353–359
- Lindenmuth GF, Lindenmuth EB (2000) The efficacy of *Echinacea* compounds herbal tea preparation on the severity and duration of upper respiratory and flu symptoms: a randomized, double blind placebo-controlled study. *J Altern Complem Med* 6:327–634
- Mark JD, Grant KL, Barton L (2001) The use of dietary supplements in pediatrics: a study of *Echinacea*. *Clin Pediatr* 40:265–269
- Mastretta C, Taghavi S, van der Lelie D, Mengoni A, Galardi F, Gonnelli C, Barac T, Boulet J, Weyens N, Vangronsveld J (2009) Endophytic bacteria from seeds of *Nicotiana tabacum* can reduce cadmium phytotoxicity. *Intern J Phytorem* 11:251–267
- Mechanda SM, Baum BR, Johnson DA, Arnason JT (2004) Sequence assessment of comigrating AFLP™ bands in *Echinacea*- implications for comparative biological studies. *Genome* 47:15–25
- Melchart D, Linde K (1999) Clinical investigations of *Echinacea* phytopharmaceutics. In: Wagner H (ed) Immunomodulatory agents from plants. Birkhäuser Verlag, Basel, Switzerland, pp 105–118
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* 15:473–497
- Pugh NP, Ross SA, ElSohly MA, Pasco DS (2001) Characterization of aloeride, a new high molecular-weight polysaccharide from *Aloe vera* with potent immunostimulatory activity. *J Agric Food Chem* 49:1030–1034
- Pugh ND, Tamta H, Balachandran P, Wu S, Howell J, Dayan FE, Pasco DS (2008) The majority of in vitro macrophage activation exhibited by extracts of some immune enhancing botanicals is due to bacterial lipoproteins and lipopolysaccharides. *Int Immunopharmacol* 8:1023–1032
- Pugh ND, Balachandran P, Lata H, Dayan FE, Joshi V, Bedir E, Makino T, Moraes R, Khan I, Pasco DS (2005) Melanin: dietary mucosal immune modulator from *Echinacea* and other botanical supplements. *Intern Immunopharm* 5:637–647
- Sauve RJ, Mmbaga MR, Zhou S (2004) In vitro propagated clones of *Echinacea tennesseensis* (Beadle) Small. *In Vitro Cell Dev Biol—Plant* 40(3):325–328
- Schweitzer JA, Bailey JK, Bangert RK, Hart SC, Whitham TG (2006) The role of plant genetics in determining above and below ground microbial communities. In: Bailey MJ, Killey AK, Timms-Wilson TM, Spencer-Phillips PTN (eds) Microbial ecology of aerial plant surfaces. CABI Internationals, Wallingford, pp 108–119
- Senchina DS, Wu L, Flinn GN, Konopka DN, McCoy JA, Widrelechner MP, Wurtele ES, Kohut ML (2006) Year-and-a-half old, dried *Echinacea* roots retain cytokine-modulating capabilities in an in vitro human older adult model of influenza vaccination. *Planta Med* 72(13):1207–1215
- Senchina DS, McCann DA, Flinn GN, Wu L, Zhai Z, Cunnick JE, Wurtele ES, Kohut ML (2009) *Echinacea tennesseensis* ethanol tinctures harbor cytokine- and proliferation-enhancing capacities. *Cytokine* 46(2):267–272
- Tamta H, Pugh ND, Balachandran P, Moraes R, Sumiyanto J, Pasco DS (2008) The majority of in vitro macrophage activation and variations in this activity exhibited by bulk commercially obtained *Echinacea* plant material is due to bacterial lipoproteins and lipopolysaccharides. *J Agric Food Chem* 56:10552–10556
- Tilburt JC, Emanuel EJ, Miller FG (2008) Does the evidence make a difference in consumer behavior? Sales of supplements before and after publication of negative research results. *J Gen Intern Med* 23:1495–1498
- Turner RB, Bauer R, Woelkart K, Hulsey TC, Gangemi JD (2005) An evaluation of *Echinacea angustifolia* in experimental rhinovirus infections. *N Engl J Med* 353:341–348
- van Oevelen S, de Wachter R, Robbrecht E, Prinsen E (2003) Induction of a crippled phenotype in *Psychotria* (Rubiaceae) upon loss of the bacterial endophyte. *Bulg J Plant Physiol* (special issue):242–247
- Vos P, Hogers R, Bleeker M, Van de Lee T, Horne M, Freijters A, Pot J, Peleman K, Kuiper M, Zabeau M (1995) AFLP: a new technique for DNA fingerprinting. *Nucleic Acid Res* 21:4407–4414